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journal or publication title	British Journal of Oral and Maxillofacial Surgery
volume	54
number	3
page range	301-305
year	2016-01-25
URL	http://hdl.handle.net/10228/00007293

doi: info:doi/10.1016/j.bjoms.2016.01.003

Available online at www.sciencedirect.com
SciVerse ScienceDirect
British Journal of Oral and Maxillofacial Surgery xxx (2015) xxx–xxx
BRITISH Journal of Oral and Maxillofacial Surgery
www.bjoms.com

Electrochemical telomerase assay for screening for oral cancer

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Accepted 3 January 2016

Abstract

Telomerase has long been known to be a marker for cancer. We have developed a new method of detecting it: the electrochemical telomerase assay (ECTA). We have previously confirmed that the assay is easier to do and more precise than the conventional telomeric repeat amplification protocol, which is currently the most widely used. Here we describe a pilot study made to establish a screening system for oral cancer using ECTA. We evaluated three types of clinical samples obtained from 44 patients with oral cancer and 26 healthy volunteers: exfoliated cells from the whole oral cavity, exfoliated cells from local lesions, and tissue from the lesion itself. The current increase ratio (Δi) obtained by ECTA was significantly higher in the oral cancer group for each type of sampling used. The threshold value for Δi was 19% when calculated by analysis of receiver-operating characteristic curves. Sensitivity and specificity values were 86% and 85% for cells from the oral cavity, 82% and 85% in cells from local lesions, and 95% and 92% in cells from the tumour itself, respectively. There were also no significant differences in sensitivity and specificity associated with age, size of tumour, site of lesion, or degree of malignancy. ECTA therefore seems to be a promising assay for screening for oral cancer.

Keywords: Telomerase; Electrochemical assay; Oral cancer screening

Introduction

Oral functions such as chewing, swallowing, and speech have important roles in daily life, and many patients who have advanced oral cancers resected are left with various dysfunctions and aesthetic problems.[1,2] Methods that enable earlier detection of oral cancer may therefore facilitate better outcomes for them by reducing the amount of resection required.

Telomerase is an enzyme that elongates the vertebrate telomere sequence TTAGGG and is associated with cellular immortalisation. Telomerase activity is present in various human cancers, but it is undetectable in most normal somatic cells.[3,4] Its presence is therefore regarded as a marker for cancer.

The telomeric repeat amplification protocol (TRAP) assay is a method of measuring telomerase activity based on a polymerase chain reaction (PCR), but is time-consuming. We have developed a new method of electrochemical telomerase assay (ECTA) to measure telomerase activity that does not require PCR or subsequent gel electrophoresis.[5–7] For ECTA a sample solution is placed on an electrode with immobilised

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http://dx.doi.org/10.1016/j.bjoms.2016.01.003

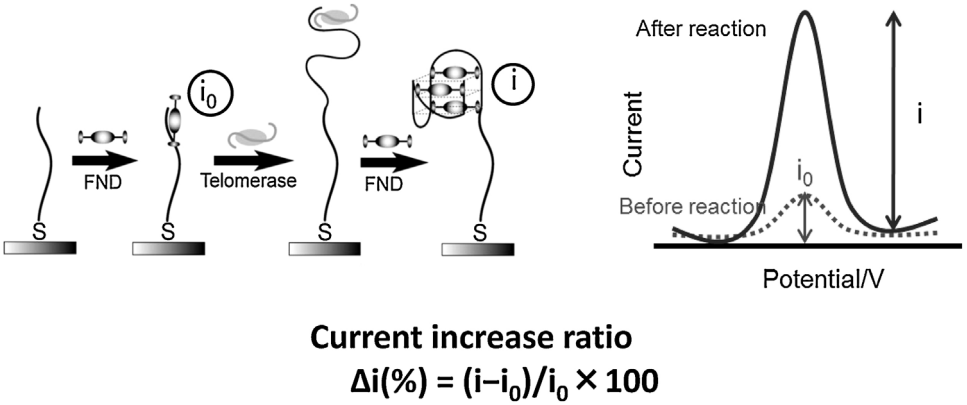

Fig. 1. Diagram of the electrochemical telomerase assay. FND= N,N'-bis[[4-(3-Ferrocenepropionamidopropyl)-piperazin-1-yl]propyl]-naphthalene- 1,4,5,8-tetracarboxylic acid diimide.

telomerase substrate primer, and the current value is measured. If it detects telomerase activity, the telomerase substrate is elongated and the current value increases. The current increase ratio (Δi) reflects the amount of telomerase activity (Fig. 1).

We previously compared the ability of ECTA and TRAP to detect telomerase activity in four cancer cell lines that were derived from oral cancers.[7] ECTA could detect telomerase activity in as little as 8 ng protein (equivalent to about 10 cancer cells), while TRAP required at least 20 times more protein. However, in ECTA the Δi value reached a plateau with relatively few cancer cells present in the sample, so ECTA is a highly sensitive assay for telomerase activity, which is qualitative but not quantitative. We then compared ECTA with TRAP in a clinical trial using both tissue and exfoliated cells scratched from the oral mucosa of patients with oral cancer, and it was vastly superior to TRAP in the detection of telomerase activity from both tissue and cells.

In the present study we aimed to establish an ECTA-based screening system for oral cancer by evaluating the reliability of ECTA in three types of clinical samples from patients with oral cancer.

Material and methods

The study was cross-sectional and done in the Department of Oral and Maxillofacial Surgery, Kyushu Dental University Hospital, Fukuoka, Japan between September 2010 and March 2013. Ethical approval was provided by the ethics committee of Kyushu Dental University (10-19). Samples were obtained from 44 patients with oral cancer and 26 healthy volunteers, all of whom gave informed consent. Patients who had previously been treated for oral cancer by excision, irradiation, or chemotherapy were excluded from the study. All patients were diagnosed histopathologically with squamous cell carcinoma (SCC).

The oral cancer group comprised 24 men and 20 women, mean age 69 (range 34–87) years. Specimens were obtained from SCC of the tongue (n=24), the gingiva (n=16), the floor of mouth (n=3), and the buccal mucosa (n=1). Tumours were sized according to the UICC criteria: T1 (< 2 cm, n=15), T2 (<4 cm but ≥2 cm, n=24), T3 (≥4 cm, n=1), and T4 (includes invasion of nearby tissue, n=4). Histopathological examination identified 27 well-differentiated, 16 moderately differentiated, and 1 poorly differentiated SCC (Table 1). The healthy volunteers were 17 men and 9 women, mean age 47 (range 24–84) years.

Samples were collected in three ways. Exfoliated cells from the whole oral cavity were collected by scratching the entire oral cavity with a sponge-type brush (2 × 2 cm). Five scratches were made on the left and right buccal mucosa, the buccal and lingual gingiva of the upper and lower jaws, and the lingual margin of the tongue. We consider this collection method suitable for use as a self-screening system for patients concerned about oral cancer. Samples of exfoliated cells from local lesions were collected by scratching a lesion with an interdental brush, as is routine for cytodiagnosis. We consider this collection method suitable for use as part of a medical examination for oral cancer by a dentist or other

Table 1
Details of patients with oral cancer.

Variable	Number
Sex:	
Male	24
Female	20
Site:	
Tongue	24
Gingiva	16
Floor of mouth	3
Buccal mucosa	1
Stage of tumour:	
T1	15
T2	24
T3	1
T4	4
Histopathological differentiation:	
Good	27
Moderate	16
Poor	1

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Table 2
Sensitivity, specificity, false positive, and false negative values for exfoliated cells from the whole oral cavity, from local lesions, and from the lesion itself.

Variable	Exfoliated cells from the oral cavity	Local exfoliated cells	Tissue from the lesion
No of patients with oral cancer	44	44	42
No of healthy volunteers	26	26	13
Sensitivity (%)	86	82	95
Specificity (%)	85	85	92
False positive rate (%)	15	15	8
False negative rate (%)	14	18	5

medical practitioner. Samples from healthy volunteers were collected from the tongue or buccal mucosa. The third method was tissue taken from the lesion. For the oral cancer group, tissue samples of about 1.5 × 1.5 × 1.5 mm were obtained by resection from the centre of the lesion. For the healthy volunteers, similar-sized samples were collected from the tongue or buccal mucosa.

Preparation and measurement of samples

All samples were homogenised in lysis buffer 500 μl (Tris-hydrochloric acid (pH 7.5) 10 mmol, magnesium chloride 1 mmol, ECTA 1 mmol, benzamidine 0.1 mmol, β-mercaptoethanol 5 mmol, 0.5% CHAPS, 10% glycerol) and stored at −80 °C. Samples from the oral cavity were resuspended in saline 20 ml and centrifuged at 20400 *g* for 5 minutes at 4 °C before homogenisation. Stored samples were thawed and spun in a microcentrifuge at 20400 *g* for 30 minutes at 4 °C before each assay, and the supernatant that contained the telomerase was collected. Protein concentrations were measured using a Proteostain Protein Quantification Kit-Wide Range (Dojindo, Kumamoto, Japan).[8] Protein concentrations were adjusted to 400 ng/μl. Whenever possible, nuclease-free buffers and reagents were used to minimize RNase contamination. After preparation, samples were kept in the same type of tube and assigned a number so that investigators were unaware of the patients' data.

Electrochemical measurements were made using a portable apparatus with a three-electrode configuration. A platinum electrode served as the counter electrode, a silver/silver chloride electrode as the reference electrode, and a telomerase substrate primer-immobilised electrode as the working electrode.[5–7] Osteryoung square wave voltammetry (SWV) values were calculated in 20 μm N,N'-bis[[4-(3-Ferrocenepropionamidopropyl)-piperazin-1-yl]propyl]-naphthalene- 1,4,5,8-tetracarboxylic acid diimide[4], acetic acid-potassium acetate 0.10 M and potassium chloride 0.10 M before and after the telomerase reaction using a procedure previously described.[5–7] For each reaction, reaction solution 20 μl (lysate 40 ng/μl, Tris-hydrochloric acid (50 mmol, pH 8.0), magnesium chloride1.0 mmol, potassium chloride 50 mmol, 2-mercaptoethanol 0.10 mmol, spermidine 0.10 mmol, and deoxynucleotide mixture 20 μmol) was placed on the electrode at 37 °C for 30 minutes. All data were standardised with Δi, which was defined as $(i/i_0 - 1) \times 100\%$, where i_0 and i refer to the current before and after the reaction, respectively (Fig. 1).

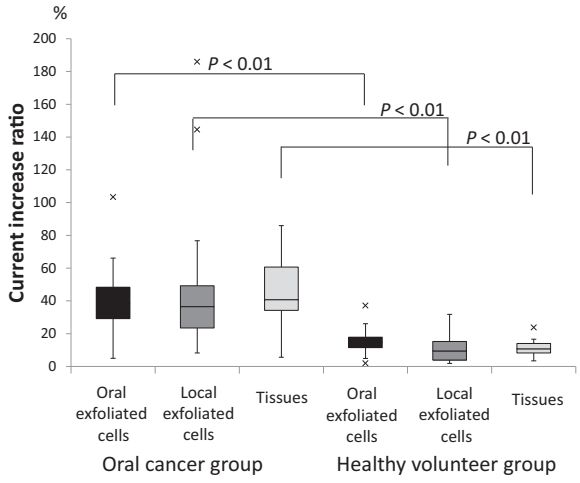

Fig. 2. Rate of increased ratio after electrochemical telomerase assay.

Analyses of data

Differences in the median values of Δi were assessed using the Mann–Whitney *U* test. Probabilities of less than 0.05 were accepted as significant. Analysis of receiver-operating characteristic (ROC) curves was used to establish threshold values, after which the sensitivity, specificity, false positive rate, and false negative rate were calculated. All statistical analyses were made with the aid of SPSS software (version 11, SPSS, Chicago, IL, USA).

Results

Within each of the three types of sample the oral cancer group had a significantly higher Δi than the healthy volunteers ($p < 0.001$; Fig. 2). Interestingly, there was no difference in the outcome of the Δi value between samples obtained from the oral cavity or the tumour itself, which might include many fewer, or many more, cancer cells, respectively.

The area under the curve for ROC curve analysis was 0.938 (Fig. 3). This analysis assigned 19%Δi as a threshold value. That is, a value less than 19%Δi was regarded as a negative result, while greater than 19%Δi was regarded as positive. We used a 19%Δi threshold value to yield sensitivity and specificity values, which confirmed that ECTA can discriminate positive and negative samples even in exfoliated cells (Table 2).

Detection of small tumours such as those classed as T1 is usually more difficult than detection of large tumours. However, the sensitivity and specificity values for ECTA

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Table 3
Sensitivity, specificity, false positive, and false negative rates in samples of cancer from exfoliated cells from the whole oral cavity, from local lesions, and from the lesion itself.

Source of cells	No of patients with oral cancer	Sensitivity (%)	Specificity (%)	False positive (%)	False negative (%)
Oral cavity*					
T stage:					
1	15	93	85	15	7
2-4	29	83	85	15	17
Site:	24	88	85	15	13
Tongue	24	88	85	15	13
Other	20	85	85	15	15
Differentiation:					
Poor/moderate	17	82	85	15	18
Good	27	89	85	15	11
Local lesions*					
T stage:					
1	15	80	85	15	20
2-4	29	83	85	15	17
Site:					
Tongue	24	83	85	15	17
Other	20	85	85	15	15
Differentiation:					
Poor/moderate	17	88	85	15	12
Good	27	78	85	15	22
The lesion itself**					
T stage:					
1	14	100	92	8	0
2-4	28	93	92	8	7
Site:					
Tongue	22	95	92	8	5
Other	20	95	92	8	5
Differentiation:					
Poor/moderate	16	94	92	8	6
Good	26	96	92	8	4

*=compared with 26 healthy volunteers and **=compared with 13 healthy volunteers.

in detecting T1 tumours did not differ from those for the detection of larger tumours (Table 3). The sensitivity and specificity of the detection of telomerase activity was the same regardless of sampling site, even for samples from the oral cavity. ECTA results did not seem to reflect the histological character of the tumours.

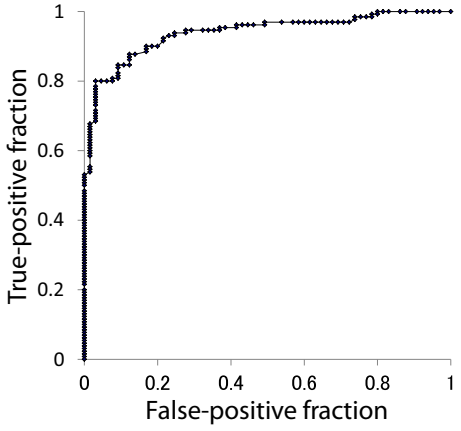

Fig. 3. Receiver-operating characteristic curve of all samples.

Discussion

It is usually possible to see and examine oral cancers. However, various mucosal diseases can impair its early detection, as neoplastic lesions are not always obvious to general dentists who are not specially trained in oral surgery or oral neoplasia. Several methods by which general dentists can detect oral cancer have therefore been reported, including vital staining with iodine[9] or toluidine blue,[10] and imaging based on autofluorescence.[11] However, interpretation of the results is subjective and often not clear, which renders them largely unreliable.

Detection of telomerase activity has been mentioned as a useful diagnostic strategy for cancer, as most cancers possess it. The conventional primer extension-based assay requires a large sample and has low sensitivity. These disadvantages were overcome by the TRAP assay established by Kim et al,[12] but that assay requires PCR and gel electrophoresis, and has the weakness that it may be impaired if inhibitors of PCR are present in samples. Saliva usually contains RNase, so contamination of a TRAP assay by RNase is unavoidable when using samples of oral origin. Assessment of telomerase activity using TRAP is by observation of the PCR amplification products after gel electrophoresis, so such judgements are also subjective and often not clear.[13]

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Because ECTA does not involve PCR, it is less influenced by RNase in saliva, and it can measure telomerase activity more quickly and precisely. Analysis of the ROC curve also indicates that ECTA has optimal discriminative ability. It is simple, and once a threshold value is established, it can be used as an ordinary objective examination that does not require a specially-trained examiner.

A similar increase in current occurred regardless of whether few (in samples from the oral cavity) or many (in samples from the tumour itself) cancer cells were present. This finding is important, as the oral cavity method of sampling enables detection of a small cancerous lesion almost anywhere within the oral cavity. It can also identify a cancer regardless of the site of onset, which could facilitate self-examination or mass examination. While ECTA did not reflect the stage of malignancy of the cancer, this is not critical for a screen, as grade can be evaluated by biopsy of lesions in patients who return a doubtful result.

One weakness of this study is the lack of comparison between ECTA and a conventional diagnostic method such as cytology using the same sample. More, larger studies are needed to validate the superiority of ECTA over well-established methods. However, these methods need specially-trained oral cytologists to interpret them or they are also subjective examinations. ECTA may be used as screening tool, however, because it is a promising objective examination that could potentially be used with samples of other body fluids such as urine, bronchoalveolar lavage, and pleural effusion.

In conclusion, we have developed a screening system for oral cancer based on measurement of telomerase activity using ECTA. We used exfoliated cells from the whole oral cavity, from a local lesion, and from samples of tissue from the tumour itself to evaluate the ability of ECTA to discriminate patients with oral cancer from healthy volunteers. ECTA could identify telomerase activity in oral cancers regardless of type of sample, size of tumour, or site of lesion, and so may well be a promising tool for screening for oral cancer.

Conflict of Interest

We have no conflict of interest.

Ethics statement/confirmation of patients' permission

This work was approved by the ethics committee of the Dental University and we obtained informed consent from all patients.

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